BANANA STREAK VIRUS PROMOTER AND DETECTION

The present invention relates to Banana Streak Virus, in particular cloning and identification of its genome and promoter, useful in transgenic strategies in *Musaceae* (*Musa* and *Ensete*) and in identification of antiviral agents, and also relates to detection of the virus in plants, in particular employing PCR primers designed on the basis of the genome sequence.

Banana (*Musa*) is the world's fourth most important commodity and is a subsistence and cash crop for many smallhold farmers particularly in West and Central Africa. Currently, improvements in the disease resistance, fruit yield and other agronomic qualities of bananas and plantains are being investigated by the International Institute for Tropical Agriculture (IITA), Nigeria, and other institutes. These improvements have been hampered by the recent diagnosis of banana streak virus (BSV) (1) in the banana breeding stocks and quarantine regulations have been preventing the movement of infected plant materials, even improved varieties.

BSV is a member of the badnavirus group (2) which have non-enveloped bacilliform particles of size 30 x 130-150 nm, containing a circular double-stranded DNA genome of 7.4-8.0 kbp. The full sequences of the genomes of four members of this group have been reported, *Commelina* yellow mottle virus (CoYMV; 3), rice tungro bacilliform virus (RTBV; 4, 5),

sugarcane bacilliform virus (ScBV; 6), cocoa swollen shoot virus (CSSV; 7) and are known for at least two other members. This information has revealed common features of the group. They all have similar genome organizations with three open reading frames (ORFs) (except RTBV, which has four) encoded on one strand. The first two ORF's potentially encode two small proteins (ORF 1 ~22 kD, ORF 2 ~ 14 kD) of unknown function. The third ORF (~210 kD) encodes a polyprotein that is proteolytically cleaved to yield the viral coat protein which contains a region with homology to an RNA binding domain together with regions with homology to aspartic protease (AP), reverse transcriptase (RT) and an RNase H (RH). In RTBV the extra ORF, ORF 4, is downstream of ORF 3 and has an unknown function. These features, together with a potential $tRNA^{met}$ binding site, suggest that badnaviruses are pararetroviruses.

Following virus entry into the cell and nucleus the genome is transcribed into a greater than genome length transcript, that (presumably) is both a polycistronic mRNA and a replication template. The DNA negative strand, primed by host $tRNA_{met}$ is synthesised by a virally-encoded reverse transcriptase, and the positive strand by the same enzyme and the virally encoded ribonuclease H.

The use of RT in their replication can potentially lead to high degree of variation between isolates and different group members, and such variation has already been reported for BSV

(8).

The present inventors have succeeded in cloning the genome of a Nigerian isolate of BSV and identifying its promoter, providing one aspect of the present invention. The promoter is useful for expression of transgenes in plants, including non-graminaceous monocots particularly *Musaceae* (*Musa* and *Ensete*) and graminaceous monocots such as rice and sugar cane. At least one strain of sugarcane bacilliform virus has been shown to infect banana (Bouhida *et al.*, 1993 *J. Gen. Virol.* **74**: 15-22).

The increasing importance of BSV highlights the need for the determination of specific properties of BSV that could be used for its diagnosis. The symptoms of BSV can be readily confused with those of cucumber mosaic virus (CMV). BSV is serologically heterogeneous (8) and is present at low titre in the host. Serological methods in use, particularly ISEM, require sophisticated equipment and are relatively insensitive. In a further aspect of the present invention, the inventors having cloned the BSV genome have designed PCR-based diagnostic systems.

PCR has been used in numerous studies for the rapid, sensitive and reliable detection of viruses from a wide variety of sources. A direct PCR protocol for the sensitive detection of BSV from *Musa* plants is reported in Harper *et al.*, 1996 (In (Ed) Marshall, *Diagnostics for Crop Protection*,

BCPC Proccedings 65n, BCPC Surrey, UK, pp 47-51). Provisional results using this PCR approach indicated widespread if not universal presence of BSV sequences in *Musa*, in contrast to results obtained using other methods, which indicated a much lower incidence of the virus. These findings and other reports provide indication of BSV sequences integrating into the *Musa* genome. The integrated sequences do no necessarily lead directly to observable disease symptoms, as there are plants documented that are and have been apparently disease free.

The present invention now provides a method that can be used for specific, reliable and sensitive detection of episomal BSV.

According to a first aspect of the present invention there is provided an isolated polynucleotide including a Banana Streak Virus (BSV) promoter. The nucleic acid may consist essentially of the promoter sequence. The promoter sequence may be part of a larger molecule including for instance a heterologous coding sequence operably linked to the promoter.

A preferred embodiment of a promoter according to the present invention has the sequence shown in SEQ ID NO 2, -350 to + 100 relative to the start of transcription site. Furthermore, a part (fragment), allele, mutant, variant or derivative of the promoter sequence shown (such as the upstream part of SEQ ID NO. 2 or a fragment thereof) should

be sufficient for promoter activity to promote transcription of a heterologous sequence operatively linked, i.e. under the control of, the part, variant or derivative of the sequence shown. One or more fragments of the sequence may be included in a promoter according to the present invention, for instance one or more motifs may be coupled to a "minimal" promoter. Such motifs may confer Banana Streak Virus promoter function on a promoter, such as suitability for or enhanced performance in non-graminaceous monocots.

In a further aspect, the present invention provides an isolated polynucleotide including a promoter, the promoter including a sequence of nucleotides shown in SEQ ID NO 2 and conferring BSV promoter function on a sequence operably linked to the promoter. Restriction enzyme or nucleases may be used to digest the full-length nucleic acid shown, followed by an appropriate assay to determine the minimal sequence required for this function. A preferred embodiment of the present invention provides a nucleic acid isolate with the minimal nucleotide sequence shown in SEQ ID NO 2 required for BSV promoter function.

The promoter may include one or more sequence motifs or elements conferring BSV promoter function regulatory control of expression. Other regulatory sequences may be included, for instance as identified by a mutation or digestion assay in an appropriate expression system or by sequence comparison with available information, e.g. using a computer to search

on-line databases.

By "promoter" is meant a sequence of nucleotides from which transcription of DNA operably linked downstream (i.e. in the 3' direction on the sense strand of double-stranded DNA) may be initiated.

"Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. DNA operably linked to a promoter is "under transcriptional initiation regulation" of the promoter.

The present invention extends to a promoter which has a nucleotide sequence which is allele, mutant, variant or derivative, by way of nucleotide addition, insertion, substitution or deletion of a promoter sequence as provided herein. Systematic or random mutagenesis of nucleic acid to make an alteration to the nucleotide sequence may be performed using any technique known to those skilled in the art. One or more alterations to a promoter sequence according to the present invention may increase or decrease promoter activity, or increase or decrease the magnitude of the effect of a substance able to modulate the promoter activity.

"Promoter activity" is used to refer to ability to initiate transcription. The level of promoter activity is

quantifiable for instance by assessment of the amount of mRNA produced by transcription from the promoter or by assessment of the amount of protein product produced by translation of mRNA produced by transcription from the promoter. The amount of a specific mRNA present in an expression system may be determined for example using specific oligonucleotides which are able to hybridise with the mRNA and which are labelled or may be used in a specific amplification reaction such as the polymerase chain reaction. Use of a reporter gene facilitates determination of promoter activity by reference to protein production.

In various embodiments of the present invention a promoter which has a sequence that is a fragment, mutant, allele, derivative or variant, by way of addition, insertion, deletion or substitution of one or more nucleotides, of the sequence of the promoter shown in SEQ ID NO 2, has homology/similarity with the shown sequence which is at least about 5% greater than the homology that any of the promoter sequences of other badnaviruses have with the sequence shown herein, preferably at least about 10% greater homology, more preferably at least about 20% homology, more preferably at least about 25% greater homology. These badnaviruses are *Commelina* yellow mottle virus, rice tungro bacilliform virus, cocoa swollen shoot virus, sugarcane bacilliform virus and Dioscorea alata bacilliform virus (unpublished data), as noted elsewhere herein. The sequence in accordance with an embodiment of the invention may hybridise with the sequence

shown in SEQ ID NO 2 but not any of the promoter sequences of these other badnaviruses under appropriately stringent selective hybridisation conditions. A promoter according to the invention may include one or more motifs that appear in SEQ ID NO 2 and are able to confer BSV promoter function on a promoter which contains them.

Similarly, nucleic acid according to certain embodiments of the present invention may have homology with all or part of the nucleotide sequence shown in SEQ ID NO 2, which homology is greater over the length of the relevant part (i.e. fragment) than the homology shared between the part of SEQ ID NO 2 and a respective part of the nucleotide sequence of any of these other badnaviruses, and may be greater than about 5% greater, more preferably greater than about 10% greater, more preferably greater than about 20% greater, and more preferably greater than about 30% greater.

Homology may be taken over the full-length of a sequence or over a part, such as 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 150, 200 contiguous nucleotides. That two nucleotide sequences are said to share "homology" or be "homologous" is based on sequence comparison. Any phylogenetic relationship is irrelevant for this. Those skilled in the art routinely refer to homology between nucleotide sequences with no implication for evolutionary origin. Two homologous nucleotide sequences may also be said to be "similar" or have a certain per centage similarity or a certain per centage

identity.

In general it is not critical which of the various standard algorithms are used to determine how homologous two nucleotide sequences are with one another. A preferred algorithm may be GAP, which uses the alignment method of Needleman and Wunsch (*J. Mol. Biol.* (1970) 48: 443-453) and is included in the Program Manual or the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA). In the absence of instructions to the contrary, the skilled person would understand to use the default parameters with the aim of maximizing alignment, with a gap creation penalty = 12 and gap extension penalty = 4.

Similarity or homology (the terms are used interchangeably) or identity may be as defined and determined by the TBLASTN program, of Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-10, or BestFit, which is part of the Wisconsin Package, Version 8, September 1994, (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA, Wisconsin 53711). Preferably sequence comparisons are made using FASTA and FASTP (see Pearson & Lipman, 1988. Methods in Enzymology 183: 63-98). Parameters are preferably set, using the default matrix, as follows: Gapopen (penalty for the first residue in a gap): -12 for proteins / -16 for DNA; Gapext (penalty for additional residues in a gap): -2 for proteins / -4 for DNA; KTUP word length: 2 for proteins / 6 for DNA.

Nucleic acid sequence homology may be determined by means of selective hybridisation between molecules under stringent conditions.

Preliminary experiments may be performed by hybridising under low stringency conditions. For probing, preferred conditions are those which are stringent enough for there to be a simple pattern with a small number of hybridisations identified as positive which can be investigated further.

For example, hybridizations may be performed, according to the method of Sambrook et al. (below) using a hybridization solution comprising: 5X SSC (wherein 'SSC' = 0.15 M sodium chloride; 0.15 M sodium citrate; pH 7), 5X Denhardt's reagent, 0.5-1.0% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes - 1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology is (Sambrook et al., 1989): T_m = 81.5°C + 16.6Log [Na+] + 0.41 (% G+C) -

0.63 (% formamide) - 600/#bp in duplex.

As an illustration of the above formula, using [Na+] = [0.368] and 50-% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C. Such a sequence would be considered substantially homologous to the nucleic acid sequence of the present invention.

It is well known in the art to increase stringency of hybridisation gradually until only a few positive clones remain. Other suitable conditions include, e.g. for detection of sequences that are about 80-90% identical, hybridization overnight at 42°C in 0.25M Na_2HPO_4, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 55°C in 0.1X SSC, 0.1% SDS. For detection of sequences that are greater than about 90% identical, suitable conditions include hybridization overnight at 65°C in 0.25M Na_2HPO_4, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 60°C in 0.1X SSC, 0.1% SDS.

An alternative, which may be particularly appropriate with plant nucleic acid preparations, is a solution of 5x SSPE (final 0.9 M NaCl, 0.05M sodium phosphate, 0.005M EDTA pH 7.7), 5X Denhardt's solution, 0.5% SDS, at 65°C overnight,

(for high stringency, highly similar sequences) or 50°C (for low stringency, less similar sequences). Washes in 0.2x SSC/0.1% SDS at 65°C for high stringency, alternatively at 50-60°C in 1x SSC/0.1%SDS for low stringency.

In a further embodiment, hybridisation of nucleic acid molecule to a variant may be determined or identified indirectly, e.g. using a nucleic acid amplification reaction, particularly the polymerase chain reaction (PCR). PCR requires the use of two primers to specifically amplify target nucleic acid, so preferably two nucleic acid molecules with sequences characteristic of BSV are employed. Using RACE PCR, only one such primer may be needed (see "PCR protocols; A Guide to Methods and Applications", Eds. Innis et al, Academic Press, New York, (1990)).

Thus a method involving use of PCR in obtaining nucleic acid according to the present invention may include:

(a) providing a preparation of nucleic acid, e.g. from a plant cell;

(b) providing a pair of nucleic acid molecule primers useful in (i.e. suitable for) PCR, at least one of said primers being a primer specific for nucleic acid according to the present invention;

(c) contacting nucleic acid in said preparation with said primers under conditions for performance of PCR;

(d) performing PCR and determining the presence or absence of an amplified PCR product.

The presence of an amplified PCR product may indicate identification of an allele or other variant.

Table 1 shows % nucleotide homologies for various Badnaviruses compared with the BSV sequence shown in SEQ ID NO 1 (over the full length) as determined using the algorithm GCG GAP (see above).

The present invention also includes promoters that are homologous to the BSV promoter, particular the sequence of SEQ ID NO 2. An homologous promoter may show greater than 55% homology with the sequence of SEQ ID NO 2, greater than 65% homology, greater than 75% homology, greater than 85% homology or greater than 95% homology. Such homology may be shown for a sequence of at least 20 nucleotide bases, at least 50 nucleotide bases, at least 100 nucleotide bases, at least 300 nucleotide bases or at least 500 nucleotide bases.

It should be noted that because banana streak virus is a retrovirus employing reverse transcriptase (RT) which is known to be relatively error prone, a certain drift is to be expected in sequences occurring in Nature. Indeed, the sequence disclosed in SEQ ID NO 2 is obtained from a Nigerian isolate of BSV but other isolates may have sequences which vary from that specific sequence to different degrees. These are encompassed by aspects and embodiments of the present invention.

An allelic variant of the BSV genome sequence of SEQ ID NO. 1 may in accordance with the present invention include one or more, or preferably all, of the following changes with respect to SEQ ID NO. 1: insertion of C as nucleotide 6779, insertion of G as nucleotide 7087 (numbered 7088 if C is inserted as nucleotide 6779), substitution of T for A at nucleotide 4218, substitution of C for G at nucleotide 6606, substitution of G for A at nucleotide 7118 (numbered 7119 if C is inserted as nucleotide 6779 or G is inserted as nucleotide 7087, and numbered 7120 if C is inserted as nucleotide 6779 and G is inserted as nucleotide 7088), substitution of C for G at nucleotide 7185 (numbered 7186 if C is inserted as nucleotide 6779 or G is inserted as nucleotide 7087, and numbered 7187 if C is inserted as nucleotide 6779 and G is inserted as nucleotide 7088), substitution of T for C at nucleotide 7207 (numbered 7208 if C is inserted as nucleotide 6779 or G is inserted as nucleotide 7087, and numbered 7209 if C is inserted as nucleotide 6779 and G is inserted as nucleotide 7088). An allelic variant of a promoter according to the invention may include one or more or preferably all of those of the above changes that fall within the promoter sequence.

Further provided by the present invention is a nucleic acid construct including a promoter region or a fragment, mutant, allele, derivative or variant thereof as discussed able to promote transcription in a plant, particularly in *Musaceae* or monocots, operably linked to a heterologous nucleic acid

sequence, preferably a gene, e.g. a coding sequence. By "heterologous" in this context is meant a gene other than any coding sequence found naturally in banana streak virus. Modified forms of BSV coding sequences may be excluded. Generally, the gene may be transcribed into mRNA which may be translated into a peptide or polypeptide product which may be detected and preferably quantitated following expression. A gene whose encoded product may be assayed following expression is termed a "reporter gene", i.e. a gene which "reports" on promoter activity.

The present invention also provides a nucleic acid vector including a promoter as disclosed herein. Such a vector may include a suitably positioned restriction site or other means for insertion into the vector of a sequence heterologous to the promoter to be operably linked thereto. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al, 1989, Cold Spring Harbor Laboratory Press. Procedures for introducing DNA into cells depend on the host used, but are well known. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are

described in detail in *Current Protocols in Molecular Biology*, Second Edition, Ausubel *et al.* eds., John Wiley & Sons, 1992. The disclosures of Sambrook *et al.* and Ausubel *et al.* are incorporated herein by reference. Specific procedures and vectors previously used with wide success upon plants are described by Bevan (Nucl. Acids Res. 12, 8711-8721 (1984)) and Guerineau and Mullineaux (1993)(Plant transformation and expression vectors. In: Plant Molecular Biology Labfax (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148).

Selectable genetic markers may be used consisting of chimaeric genes that confer selectable phenotypes such as resistance to antibiotics such as kanamycin, hygromycin, phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones and glyphosate.

A further aspect of the present invention provides a host cell (which may be microbial or plant) containing a nucleic acid construct including a promoter element, as disclosed herein, operably linked to a heterologous nucleic acid sequence or gene. A still further aspect provides a method including introducing such a construct into a host cell. The introduction may employ any available technique well known to the person skilled in the art.

The introduction may be followed by causing or allowing expression of the heterologous nucleic acid sequence or gene

under the control of the promoter.

In one embodiment, the construct including promoter and nucleic acid sequence or gene is integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by including in the construct sequences which promote recombination with the genome, in accordance with standard techniques.

Nucleic acid molecules and vectors according to the present invention may be provided isolated and/or purified from their natural environment, in substantially pure or homogeneous form, or free or substantially free of nucleic acid or genes of the species of interest or origin other than the sequence encoding a polypeptide with the required function. Nucleic acid according to the present invention may include cDNA, RNA, genomic DNA and may be wholly or partially synthetic. The term "isolate" encompasses all these possibilities. Where a DNA sequence is specified, e.g. with reference to a figure, unless context requires otherwise the RNA equivalent, with U substituted for T where it occurs, is encompassed.

An aspect of the present invention is the use of nucleic acid according to the invention in the production of a transgenic plant.

When introducing a chosen gene construct into a cell, certain considerations, well known to those skilled in the art must

be taken into account. The nucleic acid to be inserted should be assembled within a construct which contains effective regulatory elements which will drive transcription. There must be available a method of transporting the construct into the cell. Once the construct is within the cell membrane, integration into the endogenous chromosomal material either will or will not occur. Finally, as far as plants are concerned the target cell type must be such that cells can be regenerated into whole plants.

Plants transformed with the DNA segment containing the sequence may be produced by standard techniques which are already known for the genetic manipulation of plants. DNA can be transformed into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by Agrobacterium exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966, Green *et al.* (1987) *Plant Tissue and Cell Culture*, Academic Press), electroporation (EP 290395, WO 8706614) other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611), liposome mediated DNA uptake (e.g. Freeman *et al. Plant Cell Physiol.* 29: 1353 (1984)), or the vortexing method (e.g. Kindle, *PNAS U.S.A.* 87: 1228 (1990d) Physical methods for the transformation of plant cells are reviewed in Oard, 1991, *Biotech. Adv.* 9: 1-11.

Agrobacterium transformation is widely used by those skilled in the art to transform dicotyledonous and monocotyledonous species. For examples of production of stable, fertile transgenic plants in economically relevant monocot plants see *inter alia* Toriyama, et al. (1988) *Bio/Technology* 6, 1072-1074; Zhang, *et al.* (1988) *Plant Cell Rep.* 7, 379-384; Zhang, *et al.* (1988) *Theor Appl Genet* 76, 835-840; Shimamoto, *et al.* (1989) *Nature* 338, 274-276; Datta, *et al.* (1990) *Bio/Technology* 8, 736-740; Christou, *et al.* (1991) *Bio/Technology* 9, 957-962; Peng, *et al.* (1991) International Rice Research Institute, Manila, Philippines 563-574; Cao, *et al.* (1992) *Plant Cell Rep.* 11, 585-591; Li, *et al.* (1993) *Plant Cell Rep.* 12, 250-255; Rathore, *et al.* (1993) *Plant Molecular Biology* 21, 871-884; Fromm, *et al.* (1990) *Bio/Technology* 8, 833-839; Gordon-Kamm, *et al.* (1990) *Plant Cell* 2, 603-618; D'Halluin, *et al.* (1992) *Plant Cell* 4, 1495-1505; Walters, *et al.* (1992) *Plant Molecular Biology* 18, 189-200; Koziel, *et al.* (1993) *Biotechnology* 11, 194-200; Vasil, I. K. (1994) *Plant Molecular Biology* 25, 925-937; Weeks, *et al.* (1993) *Plant Physiology* 102, 1077-1084; Somers, *et al.* (1992) *Bio/Technology* 10, 1589-1594; WO92/14828. In particular, *Agrobacterium* mediated transformation is now emerging also as an highly efficient alternative transformation method in monocots (Hiei *et al.* (1994) *The Plant Journal* 6, 271-282).

The generation of fertile transgenic plants has been achieved in the cereals rice, maize, wheat, oat, and barley (reviewed

in Shimamoto, K. (1994) *Current Opinion in Biotechnology* 5, 158-162.; Vasil, *et al.* (1992) *Bio/Technology* 10, 667-674; Vain et al., 1995, *Biotechnology Advances* 13 (4): 653-671; Vasil, 1996, *Nature Biotechnology* 14 page 702).

Banana has been transformed: for example see Sagi *et al.*, 1995, *BioTechnology* 13: 481-485, and May *et al.*, 1995, *BioTechnology* 13: 486-492.

Microprojectile bombardment, electroporation and direct DNA uptake are preferred where Agrobacterium is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, eg bombardment with Agrobacterium coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with Agrobacterium (EP-A-486233).

Following transformation, a plant may be regenerated, e.g. from single cells, callus tissue or leaf discs, as is standard in the art. Almost any plant can be entirely regenerated from cells, tissues and organs of the plant. Available techniques are reviewd in Vasil et al., *Cell Culture and Somatic Cel Genetics of Plants, Vol I, II and III, Laboratory Procedures and Their Applications*, Academic Press, 1984, and Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989.

The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not essential to nor a limitation of the invention, nor is the choice of technique for plant regeneration.

Also according to the invention there is provided a plant cell having incorporated into its genome nucleic acid, particularly heterologous nucleic acid, as provided by the present invention. A further aspect of the present invention provides a method of making such a plant cell involving introduction of a vector including the sequence of nucleotides into a plant cell and causing or allowing recombination between the vector and the plant cell genome to introduce the sequence of nucleotides into the genome. The invention extends to plant cells containing nucleic acid according to the invention as a result of introduction of the nucleic acid into an ancestor cell.

A further aspect of the present invention provides a method of making such a plant cell involving introduction of nucleic acid or a suitable vector including the sequence of nucleotides into a plant cell and causing or allowing recombination between the vector and the plant cell genome to

introduce the sequence of nucleotides into the genome. The invention extends to plant cells containing nucleic acid according to the invention as a result of introduction of the nucleic acid into an ancestor cell.

In the context of a host cell containing "heterologous" nucleic acid, the term "heterologous" may be used to indicate that the gene/sequence of nucleotides in question have been introduced into said cells of the plant or an ancestor thereof, using genetic engineering, i.e. by human intervention. A transgenic plant cell, i.e. transgenic for the nucleic acid in question, may be provided. The transgene may be on an extra-genomic vector or incorporated, preferably stably, into the genome. A heterologous gene may replace an endogenous equivalent gene, ie one which normally performs the same or a similar function, or the inserted sequence may be additional to the endogenous gene or other sequence. An advantage of introduction of a heterologous gene is the ability to place expression of a sequence under the control of a promoter of choice, in order to be able to influence expression according to preference. Furthermore, mutants, variants and derivatives of the wild-type gene, e.g. with higher or lower activity than wild-type, may be used in place of the endogenous gene. Nucleic acid heterologous, or exogenous or foreign, to a plant cell may be non-naturally occuring in cells of that type, variety or species. Thus, nucleic acid may include a coding sequence of or derived from a particular type of plant cell or species or variety of

plant, placed within the context of a plant cell of a different type or species or variety of plant. A further possibility is for a nucleic acid sequence to be placed within a cell in which it or a homologue is found naturally, but wherein the nucleic acid sequence is linked and/or adjacent to nucleic acid which does not occur naturally within the cell, or cells of that type or species or variety of plant, such as operably linked to one or more regulatory sequences, such as a promoter sequence, for control of expression. A sequence within a plant or other host cell may be identifiably heterologous, exogenous or foreign.

Plants which include a plant cell according to the invention are also provided, along with any part or propagule thereof, seed, selfed or hybrid progeny and descendants. A plant according to the present invention may be one which does not breed true in one or more properties. Plant varieties may be excluded, particularly registrable plant varieties according to Plant Breeders' Rights. It is noted that a plant need not be considered a "plant variety" simply because it contains stably within its genome a transgene, introduced into a cell of the plant or an ancestor thereof.

In addition to a plant, the present invention provides any clone of such a plant, seed, selfed or hybrid progeny and descendants, and any part of any of these, such as cuttings, seed. The invention provides any plant propagule, that is any part which may be used in reproduction or propagation,

sexual or asexual, including cuttings, seed and so on. Also encompassed by the invention is a plant which is a sexually or asexually propagated off-spring, clone or descendant of such a plant, or any part or propagule of said plant, off-spring, clone or descendant.

The invention further provides a method of influencing or affecting a physical characteristic of a plant, including causing or allowing expression of a heterologous nucleic acid sequence as discussed within cells of the plant. Characteristics which may be influenced include resistance, immunity, tolerance, hypersensitivy to pathogens such as viruses, fungi and bacteria, pests such as nematodes and weevils, agronomic characters such as dwarfism of the plant, yield of seed or other product, fertility or sterility and quality of fruit.

The invention further provides a method of influencing a physical characteristic of a plant including expression from nucleic acid according to the present invention as above, within cells of a plant, following an earlier step of introduction of the nucleic acid into a cell of the plant or an ancestor thereof. Such a method may influence or affect a characteristic of the plant, such as noted above. This may be used in combination with any other gene, such as transgenes involved in determining or modifying any of the characteristics noted or other phenotypic trait or desirable property.

Nucleic acid constructs including a promoter (as disclosed herein) and a heterologous gene (reporter) may be employed in screening for a substance able to modulate activity of the promoter. For anti-viral purposes, e.g. for treatment of BSV in banana or other disease, a substance able to down-regulate expression of the promoter may be sought. In other contexts, e.g. for expression of a product useful in modifying a plant characteristic as noted, it may be desirable to obtain a substance able to up-regulate expression from the promoter. A method of screening for ability of a substance to modulate activity of a promoter may include contacting an expression system, such as a host cell, containing a nucleic acid construct as herein disclosed with a test or candidate substance and determining expression of the heterologous gene.

The level of expression in the presence of the test substance may be compared with the level of expression in the absence of the test substance. A difference in expression in the presence of the test substance indicates ability of the substance to modulate gene expression. An increase in expression of the heterologous gene compared with expression of another gene not linked to a promoter as disclosed herein indicates specificity of the substance for modulation of the promoter.

A promoter construct may be introduced into a cell line using any technique previously described to produce a stable cell

line containing the reporter construct integrated into the genome. The cells may be grown and incubated with test compounds for varying times. Cells may be regenerated into plants and assay or screening methods carried out in accordance with the present invention on the plant or a part thereof, such as leaf or fruit.

The cells and/or plant may be *Musaceae*, *Musa*, *Ensete*, banana or plantain.

Following identification of a substance which modulates or affects promoter activity, the substance may be investigated further. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition which may contain at least one additional component, such as a diluent or solvent. These may be administered to cells or plants to modulate promoter activity.

Thus, the present invention extends in various aspects not only to a substance identified using a nucleic acid molecule as a modulator of promoter activity, in accordance with what is disclosed herein, but also a composition including such a substance, a method including administration of such a composition to a plant, e.g. for decreasing expression for instance in treatment (which may include preventative treatment) of BSV or other disease of a plant, such as of *Musa*ceae (*Musa* or *Ensete*) or monocots such as noted above,

use of such a substance in manufacture of such a composition and a method of making a composition including admixing such a substance with an acceptable diluent or carrier, and optionally other ingredients.

Further aspects of the present invention relate to "diagnostic" methods and means for determining the presence in a plant, particularly *Musaceae*, of episomal banana streak virus.

According to a further aspect of the present invention there is provided a method of determining the presence or absence of banana streak virus in a plant, the method including

bringing into contact specific binding molecules specific for banana streak virus and a test sample including extract of a test plant under conditions wherein the specific binding molecules bind banana streak virus particles if present in the test sample;

performing PCR on the test sample using primers specific for banana streak virus;

determining the presence or absence of a PCR product characteristic of banana streak virus.

The plant extract to be tested may be provided by using a carbonate buffer as grinding medium, as is well known in the art.

The specific binding molecules may be immobilised on a solid

support such as a column, allowing for ease of washing away of non-binding material (e.g. Clark *et al.* (1986) *Methods in Enzymology* 118: 742-751). One or more washing steps is preferably included prior to performance of the PCR.

The specific binding molecules may include antibodies or specific binding fragments thereof.

Methods of producing antibodies include immunising a mammal (e.g. human, mouse, rat, rabbit, horse, goat, sheep or monkey) with banana streak virus or one or more proteins or protein fragments thereof. Antibodies may be obtained from immunised animals using any of a variety of techniques known in the art, and might be screened, preferably using binding of antibody to antigen of interest. For instance, Western blotting techniques or immunoprecipitation may be used (Armitage et al, 1992, Nature 357: 80-82). Antibodies may be polyclonal or monoclonal.

As an alternative or supplement to immunising a mammal, antibodies with appropriate binding specificity may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047.

Various fragments of antibodies are known in the art to have

the ability to bind target antigen specifically, including Fab, scFv, Fd, Fv, diabodies and so on. Since the role of a specific binding member in the context of this aspect of the present invention is to bind, and effector functions provided by whole antibody are not relevant, any specific binding molecule including any binding fragment of an antibody may in principle be used.

Suitable PCR primers include those which are directed to regions of the genome that are likely to be conserved among BSV isolates, such as in sequences coding for amino acid sequences conserved among plant pararetroviruses in the aspartate protease and reverse transcriptase regions. Because of the redundancy of the genetic code, these sequences are not found in other pararetroviruses.

Preferred primers include the following sequences:
Forward v3012, 5' GGA ATG AAA GAG CAG GCC
Reverse v 1573, 5' AGT CAT TGG GTC AAC CTC TGT CCC.

PCR techniques for the amplification of nucleic acid are described in US Patent No. 4,683,195. In general, such techniques require that sequence information from the ends of the target sequence is known to allow suitable forward and reverse oligonucleotide primers to be designed to be identical or similar to the polynucleotide sequence that is the target for the amplification. PCR comprises steps of denaturation of template nucleic acid (if double-stranded),

annealing of primer to target, and polymerisation. The nucleic acid sequence information provided herein, particularly in SEQ ID NO 1, readily allow the skilled person to design PCR primers. References for the general use of PCR techniques include Mullis et al, Cold Spring Harbor Symp. Quant. Biol., 51:263, (1987), Ehrlich (ed), PCR technology, Stockton Press, NY, 1989, Ehrlich et al, Science, 252:1643-1650, (1991), "PCR protocols; A Guide to Methods and Applications", Eds. Innis et al, Academic Press, New York, (1990).

On the basis of the sequence and other information provided herein, oligonucleotide primers may be designed by those skilled in the art. An oligonucleotide for use in nucleic acid amplification may have about 10 or fewer triplets (e.g. 6, 7 or 8), i.e. be about 30 or fewer nucleotides in length (e.g. 18, 21 or 24). Generally specific primers are upwards of 14 nucleotides in length, but need not be more than 18-20.

The presence of a PCR product indicative or characteristic of banana streak virus may be determined by means of any of the many screening procedures available to those skilled in the art, including detection of the PCR products by size, hybridization with a BSV probe, restriction endonuclease restriction patterns and/or sequencing.

Further aspects and embodiments will be apparent to those of ordinary skill in the art. Embodiments of the present

invention will now be illustrated by way of example.

Figure 1 shows a map of the BSV genome, the full sequence for which is shown as SEQ ID NO. 1.

SEQ ID NO. 2 shows a sequence of a preferred promoter according to one embodiment of the present invention.

All documents mentioned anywhere herein are incorporated by reference.

EXAMPLE 1 - Cloning of BSV

Virus isolation and purification

BSV-infected banana plants (cultivar TMP4698 which is a tetraploid hybrid of Obino l'Ewai x Calcutta 4) were supplied, as *in vitro* plantlets or screenhouse-grown plant suckers, by IITA, Onne Field Station, Nigeria. Plant sucker outer tissue was removed and the corms were sterilised in 1% sodium hypochlorite for 1 h and quarantined for 1 month, prior to moving to the greenhouse and maintenance at 28°C day, 25°C night.

Leaf material was finely ground in liquid nitrogen and blended into 2 volumes buffer A (50 mM sodium phosphate pH 6.1, 5 mM dithiothreitol, 5 mM diethyldithiocarbamate, 0.5% polyethylene glycol (PEG 6000). Celluclast (Novo Nordisk) was added to 2%, incubated with stirring at 37°C for 2 h then

overnight at room temperature. Triton X-100 was added to 1% and the incubation carried out for a further 30 min. All subsequent steps were carried out at 4°C. The supernatant from a low speed centrifugation at 10,000g for 10 min, was further centrifuged at 120,000g for 90 min. The pellet was resuspended in 100 ml buffer A, centrifuged through a 5% sucrose cushion at 120,000g for 2.5 h and the pellet resuspended in 5 ml buffer A. The virus was further purified according to Lockhart (2) by centrifugation in a 0-40% Cs_2SO_4 gradient in 10% sucrose [steps 40, 30, 20, 10, 0%] at 120,000g for 2.5 h. The viral band was identified by ISEM, carried out according to Lockhart (2) using mixed BSV antisera. The virus was diluted four fold in buffer A, pelleted at 150,000g for 60 min and resuspended in 100 µl 50 mM sodium phosphate pH 6.1.

Cloning and sequence analysis

Virion DNA was purified by digestion of virus particles with Proteinase K at 1 mg/ml in 100 mM TrisCl pH 8.0, 2 mM $CaCl_2$, 2% SDS, for 2 h at 65°C. Following phenol extraction, the DNA was precipitated, washed in 70% ethanol, dried and resuspended in 50 µl TE [10 mM TrisCl, 1 mM EDTA pH 8.0) Sambrook *et al.* (13)]. The DNA was digested with *Eco* R1 and the resulting fragments cloned into pBluescript II SK+ (Stratagene). One clone had sequence homology to ScBV and other badnaviruses. Primers were designed using the clone sequence information to allow PCR amplification of the entire virion DNA. The primers were contiguous, facing in opposite

directions (nucleotide numbering for the completed BSV sequence, see below): V1514 5' TGCGGGTGCTTCTTCACCC (anti-sense @ 2778), V1517 5' TATGCACCAGCTACAAGTGC (sense @ 2779). The Expand™ Long Template PCR system (Boehringer) was used, following the manufacturer's protocol for a 0.5-12 kb template (system 1). The template was 0.1-1.0 µl of the isolated virus DNA and the primers were used at a final concentration of 300 nM. The amplification cycle conditions were 94°C x 1 min, [92°C x 20 s, 50°C x 30 s, 68°C x 6 min] x10, [92°C x 20 s, 50°C x 30 s, 68°C x 6 min with 20 s increment/cycle] x 15 and a final extension for 68°C x 7 min.

The 7.39 kb PCR product and sub-clones derived from it were sequenced manually using Sequenase version 2.0 (USB, United States Biochemicals) and automatically using the Prism system (Applied Biosystems) and an ABI 373 sequencer. The sequence was analysed using the GCG sequence package (14). All DNA manipulations were performed according to Sambrook *et al.* (13).

For isolation of RNA, 2 g symptomatic banana leaves were ground to a powder in liquid N_2, added to 24 ml 100 mM Tris-Cl pH 8.0, 20 mM EDTA, 500 mM NaCl, 20 mM mercaptoethanol, 2% SDS and incubated at 65°C for 10 min. After the addition of 8 ml 3M sodium acetate and incubation on ice for 10 min, the solution was centrifuged at 10,000 x g for 10 min and the supernatant was filtered through two layers of Miracloth. The nucleic acid was precipitated by the addition of 0.7 vol.

isopropanol, incubation at -20°C for 30 min and centrifugation. The pellet was resuspended in 2 ml TE, extracted with phenol/chloroform then chloroform and the RNA precipitated by the addition of lithium acetate to give 2 M and incubation at 4°C overnight. The RNA pellet was washed twice in 80% ethanol and resuspended in 40 µl water. Formaldehyde-containing gel electrophoresis and northern blotting of RNA was as described in Sambrook *et al.*(13). The 5′ end of the transcript was mapped by primer extension using the method of Medberry *et al.* (3). The primer used was 5-ATCTTGCGCTCTACTCGC at 7361 bp in the BSV sequence.

Primer design and PCR

PCR primer pairs were chosen from aligned amino acid sequences corresponding to the aspartic protease and reverse transcriptase regions of the derived BSV sequences. The PCR was performed on DNA isolated from banana leaves using the method of Li *et al.* (15), using the basic protocol described by manufacturers of the *Taq* DNA polymerase (Gibco BRL). The conditions were 94°C for 2 min, [94°C for 1 min, 40-50°C for 1 min, 72°C for 1 min] x 30 cycles, followed by a 5 min extension at 72°C. Reaction products were separated by electrophoresis through a 1.5% agarose gel and detected by fluorescence of ethidium bromide under UV light. Following photography, the reaction products were transferred onto nylon membrane (Hybond-N, Amersham). A ^{32}P-labelled probe was synthesised by random priming using the full-length BSV PCR product as template (16). Hybridisation and washing was at

65°C using the solutions and protocols described by the membrane supplier (Amersham).

Virus purification

The virus purification gave a low yield of bacilliform particles of size ~30 x 130 nm, that could be trapped on BSV antiserum-coated carbon grids but not on CSSV antiserum-coated grids. The virus could be detected by ISEM in a crude extract of infected leaf, but not in leaf-dip preparations.

Virus sequence analysis

The complete 7388bp nucleotide sequence of both strands of the Nigerian isolate of BSV was determined as is shown in SEQ ID NO 1. As with other badnaviruses and caulimoviruses the numbering of the sequence begins at the putative 5' minus-strand priming site and this conserved $tRNA^{met}_{init}$ binding site sequence is found in BSV.

Overall the sequence shows low but recognisable similarity to other badnaviruses, with highest similarity to ScBV and least to RTBV (Table 1). The sequence is sufficiently different from those of other badnaviruses for BSV to be considered a distinct virus.

The + strand contains three large ORF's (Figure 1). This number, their size and order are similar to other badnavirus sequences, with the exception of RTBV which possesses an additional ORF (4, 5). The BSV ORF III shows similarity to

the ORF III sequences of other badnaviruses, in particular over regions that encode the conserved putative viral replicase functions AP, RT and RH.

Transcript mapping

Northern blots of total RNA from BSV-infected banana tissue revealed only one BSV-specific band of 7.5 kb. This is the size expected for the major transcript of a pararetrovirus the size of BSV.

To map the 5' end of the major transcript RNA extracted from infected banana tissue was used for primer extension. The primer was 5'-ATCTTGCGCTCTACTCGC-3'. Two strong stops were found indicating that the transcript start site was at 7260 or 7261bp, 25 nt downstream of a potential TATA box (TATATAA) sequence (see later). Attempts to map the 3' end of the transcript using the approach of Medberry *et al.* (3) proved unsuccessful.

PCR detection of BSV

Primer sequences for PCR were selected from within the conserved sequences putatively coding for RT and AP. This primer pair consistently gave a strong amplification product, of the expected size of 644 bp. Hybridisation with a BSV probe and sequencing of this product confirmed its origin as BSV. Amplification of this band could also be achieved from crude preparations from infected plants. All *Musa* plants tested gave this same size band, and all plants from Onne

station gave the same pattern when the band was cut with *Alu* 1.

DISCUSSION

The BSV genome contains three large ORF'S (Figure 2) potentially coding for proteins of 20.8, 14.5 and 208 kD. The size and order of the ORF'S are very similar to those of most other badnaviruses. Characteristically the ORF'S overlap, that between ORFs 1 and 2 being ATGA (ATG being start of ORF 2 and TGA stop of ORF 1) and thus resembling RTBV (Hay *et al*) and that between ORFs 2 and 3 being TAATG. Badnaviruses are characterised by having very few AUG translational start codons in the first two ORFs (see reference 16). Fütterer *et al*. (17) proposed that ORFs 2 and 3 of RTBV (and other badnaviruses) are translated by leaky scanning due to the paucity or poor context of start codons in ORFs 1 and 2. It was also suggested that the AUG-rich leader sequence is bypassed, at least in RTBV, by a "ribosome-shunt" mechanism (18). The leader sequence of BSV is rich in AUG codons. ORF 1 has four AUG codons which is more than other badnaviruses but they are all in poor context (19); the sole AUG codon in ORF 2, that at the start, is also in a poor context. Thus, although there are more AUG codons than other badnaviruses in ORF 1 it is likely that the expression strategy is similar to that described by Fütterer *et al*. (17).

The presence and arrangement of functional domains in ORF

III, cysteine rich zinc finger-like RNA-binding domain common to all retroelements (20), AP, RT and RH found in ORF III is identical to the those found in other badnaviruses (Table 2). This ORF also contains the additional cysteine-motif characteristic of all other badnaviruses thus far sequenced. The function of this extra "cys" sequence is unknown but it may be involved with translational control (21).

Phylogenetic analysis of the currently available badnavirus sequences shows BSV to be more closely related to ScBV and the other "three ORF" badnavirus than to RTBV. The sequence is sufficiently different from those of other badnaviruses for it to be considered a distinct virus.

The 5' end of the transcript was mapped to the cytosine/adenosine doublet at 7260/7261, a similar relative position in the genome as in CoYMV (3) and at +25nt with respect to a potential TATA box. There is also an *as-1*-like sequence as described by Medberry *et al* (3) for CoYMV (23) (Table 2). A potential polyadenylation signal (AAATAAAAA) is found at 7295 bp. which would give a 35 nt terminal redundancy to the full-length transcript . This terminal redundancy is smaller than those of other plant pararetroviruses (e.g. CoYMV 109-132 nt, RTBV 215 - 216 nt; CaMV 176 nt ; 3, 23, 23) but of similar size to some of the retrovirus terminal redundancies (mouse mammary tumor virus, 15 nt; avian leukosis virus 20 nt; 24). Although it was not possible to map the 3' end of the BSV transcript it cannot be

downstream of the tRNA binding site as this would affect the replication mechanism. The 5' end of the transcript is 128/127 nt from the tRNA binding site but no polyadenylation signal motifs were found downstream of that at 7361.

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EXAMPLE 2 - Expression of a heterologous sequence operably linked to BSV promoter

All standard methods of molecular biology are from Ausubel *et al.*, Current Protocols in Molecular Biology, Wiley, 1996.

Subcloning the BSV promoter/leader fragment

A portion of the BSV genome (fragment length of 1614 bp), was amplified from plasmid pSac21 by polymerase chain reaction with the addition of suitable cloning sites for creation of a chimeric gene.

The primer defining the 5' end of the promoter/leader element was 5'-GCCAGATCTAAGCTTCCCGGGATAATCAGAACTGACAGTCA-3'.

The primer defining the 3' end of the promoter/leader element was 3'-GCCCCATGGATTGTATGCAAGGTGAA-3'.

This region of the BSV genome comprises the promoter and leader (pBSV/BSV1) for expression of BSV genes. BglII and SmaI sites were added at the upstream end of the promoter element, and an NcoI site was added at the end of the BSV leader region. This fragment was cloned blunt ended into the EcoRV site of pBluescriptKS+ (Stratagene, La Jolla, CA, USA), and several clones were sequenced to identify one with the correct sequence.

Construction of pBH850

To prepare a LUCintron chimeric gene, an overlap PCR strategy was used, in which three different fragments were generated as follows:

1) fragment (1) amplification of pJJ3792 (source of LUC) with primer set NC389(1) and NC392(4) to give an upstream portion of the LUC CDS overlapping fragment (2);

2) fragment (2) amplification of pAR4401 (source of st-ls ivs2 intron, described in Vancanneyt et al., (Mol Gen Genet, 220, 245-50, 1990)) with primers NC390(2) and NC393(5) to give the st-lS ivs2 intron overlapping fragment (1);

3) fragment (3) amplification of pJJ3792 with primers NC391(3) and NC394(6) to give a downstream portion of the LUC cds overlapping fragment (2).

All reactions were done with Pfu polymerase using 20 cycles. Subsequently, 1μl of fragment (1) and 1μl of fragment (2) were mixed and amplified using Taq polymerase (15 cycles) with primers NC389 and NC393.

In a separate reaction, 1μl of fragment (2) and 1μl of fragment (3) were amplified under the same conditions using primers NC390 and NC394 to produce fragments (1-2) and (2-3). In the final reaction, 1μl of fragment (1-2) and 1μl of fragment (2-3) were mixed and amplified using Pfu polymerase (15 cycles) to generate the full length product using primers NC389 and NC394. This fragment was cloned into a pUC-based vector as a blunt-ended fragment, and the sequence of an isolate confirmed.

Primers:

NC389

5'-AACCATGGAAGACGCCAAAAACATAAAGAAAGGCCCGGCGCCATTCTATCCCCT-3'

NC390

5'-GTAAGTTTCTGCTTCTACCTT-3'

NC391

5'-GTGGCCCCCGCTGAATTGGAA-3'

NC392

5'-AGGTAGAGCAGAAACTTACCTGATACCCTTTGTATTTA-3'

NC393

5'-TTCCAATTCAGCGGGGGCCACCTGCACATCAACAAATTT-3'

NC394

5'-ACTCTAGATTACAATAGCTAA-3'

The resulting LUC-int sequence that includes the complete luciferase CDS was used to construct pBH850, which has the LUCint sequence linked to the SpMas promoter (construct 6 from Ni et al., Plant J., 7, 661-76, 1995) and the nos 3' end (Bevan et al., Nuc Acids Res, 11, 369-85, 1983). The LUC-int sequence was isolated as an NcoI-XbaI fragment, following digestion and gel purification. A pUC plasmid that had a LUC sequence (de Wet et al., Mol Cell Biol, 7, 725-37, 1987) linked to the SpMas promoter and the nos 3' end was digested with NcoI and XbaI and the fragment containing the vector, promoter and 3' end was gel-purified. These two fragments were ligated together to give pBH850, which comprises a SpMas-LUC-int-3'nos chimeric gene.

Construction of a pBSV/BSV1-LUC chimeric gene

The pBSV/BSV1 fragment was excised from the plasmid described above using XhoI and NcoI, and the fragment was gel-purified. A pUC-based plasmid (pBH850) containing a SpMas-LUCintron-3'nos chimeric gene was digested with PstI and NcoI to liberate a fragment containing the LUC-INT CDS and the nos 3' end, which was gel-purified. pBluescriptKS+ was digested with PstI and XhoI, and the vector fragment was gel-purified. The three fragments were ligated together to create a pBluescript-derived plasmid with the BSV/BSV/LUC-INT/3'nos chimeric gene. Plasmid isolates with the expected restriction digestion pattern were identified. One such isolate was designated pMM7393 and retained for transient expression analysis. Plasmid DNA was prepared using a standard alkaline lysis method.

Expression reference plasmid

As a reference plasmid for the transient expression analysis, a pUC-based plasmid (pJJ3792; available from DNA Plant Technologies, Oakland, CA, USA) containing a CaMVp35S-LUC-3'nos chimeric gene was used. Plasmid DNA was prepared using a standard alkaline lysis method.

Biollistics control plasmid

As an internal control for shot-to-shot variation for biollistics DNA delivery, a pUC-based plasmid (pP07119) comprising a 2xCaMVp35S-GUSintron-3'ocs chimeric gene was used. This is one of a wide range of similar plasmids that

can be used as a biollistics control for banana, including plasmids such as pZO1052 (which has a CaMV 35S-GUS chimeric gene; Ha et al., Plant Cell Rep, 11, 601-604, 1992) or pBI221 (which has a similar chimeric gene; Clontech, Palo Alto, CA). Plasmid DNA was prepared using a standard CsCl gradient method.

Biollistics DNA delivery

The banana suspension cultures were derived from male flowers of the cv. Grand Nain, and maintained in liquid according to Grapin et al. (In Vitro Cell. Dev. Biol.-Plant, 32, 66-71, 1996). Suspension cells were removed from fresh suspension cultures, the suspension cells were successively sieved through nylon mesh to produce a fraction between 250μm and 600μm, and the embryogenic suspension fraction was then spread thinly onto filter paper discs. Approximately 20-30mg of suspension cells were used per biollistics shot.

Plasmid DNA was precipitated onto tungsten particles using a standard CaC12/spermidine precipitation step (Vain et al., Plant Cell Rep., 12, 84-88, 1993). 20μg of each LUC-gene plasmid were mixed with 10μg of the GUS control plasmid and coated onto 2mg of tungsten particles. 3μl of suspended tungsten particles (out of 15μl in a single prep) were delivered per shot, using a particle inflow gun (Finer et al., Plant Cell Rep., 11, 323-28, 1992).

Enzymes assays

The suspension cells were incubated for 19 hours and then luciferase activity (resulting from expression of the LUC chimeric gene) was monitored using a single photon counting program on a Beckman scintillation counter. Beta-glucuronidase activity (resulting from expression of the GUS chimeric gene) was measured by determining fluorescence of the released methyl-umbelliferone from the methylumbelliferyl-glucuronide substrate. 12 DNA introductions were performed with each prep; 8 of the samples were taken for luciferase assay, and 4 of the samples were taken for glucuronidase assay.

Results

Results demonstrating good expression driven by BSV promoter *in planta* are shown in Table 4 below.

EXAMPLE 3 - Detection of BSV

Virus Isolation

Musa plants with typical chlorotic streaks were collected from the Onne Research Station, IITA, Nigeria. Virus minipreps were prepared from 5 g leaf tissue samples following the protocol of Ahlawat *et al.* (1996). The identity of the BSV was confirmed by ISEM.

DNA extraction and sample preparation

DNA was isolated from *Musa* leaf tissue by the method of Li *et*

al. (1994). Crude extracts were prepared from growing leaves for direct PCR and IC-PCR by grinding at 1:10 (w/v) in either of two different protocols. The first used 1 x phosphate-buffered saline (PBS) / 10 mM mercaptoethanol as grinding medium, followed by centrifugation at 13,000 g for 1 min. The supernatant was removed and used in the subsequent assay. The alternative used PBS/ 2% poly-vinyl pyrrolidine (PVP) /1% sodium sulphite as grinding medium followed by filtration through glasswool. The resulting filtrate was used.

For DB-PCR, plant extracts were prepared by the second method using carbonate buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, per litre, pH 9.6) as grinding medium.

PCR and primers

The PCR reaction mix of 50 µl contained 1x buffer supplied (Gibco-BRL), 200 µM each dATP, dCTP, dGTP, dTTP, 2.5 mM $MgCl_2$, 10 pmol each forward and reverse primer, 2 units *Taq* polymerase (Gibco-BRL). PCR conditions were 94°C x 1 min, (94°C x 1 min, 50°C x 1 min, 72° x 2 min) x 33 cycles and a final extension at 72°C°C x 5 min. Primers used to detect BSV were the pair v3012-v1573 of which the sequences are given above. They were chosen from aligned amino acid sequences corresponding to the AP and RT regions derived from the Onne isolate BSV sequence. The primers have been tested for the amplification of a predicted 644 bp product and its specificity to BSV has been confirmed by Southern hybridization and sequence analysis of the product (Harp r et

al., 1996). *Musa* nuclear DNA was detected by PCR using the primer pair 5'ACTAAAACGCCTATAACTCC and 5' GCTCCAATACCCATAAGAA to amplify a 260 bp fragment of a *Musa* intermediate repetitive sequence (Baurens *et al.* 1996).

A region of the nuclear encoded ACC oxidase gene was amplified using degenerate primers to two conserved regions ASFYNPGS (forward 5'GCGTCGTTCTAYAACCCGGRAGC) and EPRFEAM (reverse 5' SCYWNCGAAKCTT GGMWCC). EMBL accession x91076, *Musa acuminata* acco1 gene.

Mitochondrial DNA was detected using the primer pair *nad*1B-*nad*1C to amplify an intron sequence of the *nad*1 mitochondrial gene (Demesure *et al.* 1995). Chloroplast DNA was detected by PCR using the primer pair *rbcL*P1-*atpB*P1 to amplify the intergenic region between *rbcL* and *atpB* (Al-Janabi *et al.* 1994).

IC-PCR and DB-PCR

For IC-PCR, rabbit polyclonal antiserum to BSV or a mouse polyclonal antiserum to BSV were used for coating. Sterile 0.5 ml polypropylene microcentrifuge tubes were loaded with 25 µl of purified IgG (1 ug/µl diluted to 1/500) in carbonate buffer, and incubated at 37°C for 2 hours or 4°C overnight. After 3 washes with PBS/0.05% Tween-20, 25 µl plant extract were loaded and incubated at 37°C for 2 hours or 4°C overnight. For DB-PCR the plant extract in coating buffer was added directly to the tubes without prior coating with anti-

BSV IgG and incubated at 4°C overnight. For both techniques the tubes were similarly washed with PBS three times and dried briefly. PCR was carried out directly in the tubes, as described for PCR, without a specific disruption treatment for the virion particles.

Analysis and RE analysis of PCR products

After amplification, 10-15 µl of the PCR reaction products were separated by electrophoresis through 1.2 % agarose in either 1x TBE or 1x TAE. Following ethidium bromide binding DNA was visualised on a UV transilluminator. DNA markers were supplied by Gibco-BRL. PCR products were directly restricted in the PCR reaction mix by the addition of 0.5 µl of the appropriate restriction enzyme to 10 µl of each PCR reaction and incubating at 37°C for 1 hour prior to electrophoresis of the whole sample.

Southern hybridisation

Reaction products were separated by electrophoresis through a 1.5% agarose gel and detected by fluorescence of ethidium bromide under UV light. Following photography, the reaction products were transferred onto nylon membrane (Hybond-N, Amersham). A ^{32}P-labelled probe was synthesised by random priming using the full-length BSV PCR product as template (Feinberg and Vogelstein 1983). Hybridisation and stringent washing was carried out at 65°C using the solutions and protocols described by the membrane supplier. Autoradiography was carried out as described by Sambrook *et al.* (1989)

Cloning and sequencing

PCR products were cloned into a TA vector (Invitrogen) following purification with PCR preps kit according to the manufacturers protocol (Promega). All DNA manipulations were performed according to Sambrook *et al.* (1989). Plasmids containing the cloned PCR product inserts were sequenced by the dideoxynucleotide chain termination method (Sanger *et al.* 1977) using Sequenase version 2.0. (United States Biochemicals) and M13 forward and reverse primers.

Results

Standard-PCR and amplification conditions

The expected BSV DNA fragment of 664 bp was amplified from leaf tissue samples collected from field grown plants with or without characteristic BSV chlorotic or necrotic streak symptoms. The same amplification product was also obtained from leaf samples collected from plants grown in a screenhouse or cool room (22°C) again with or without BSV symptoms. More interestingly, the expected amplified product was obtained from tissues collected from *in vitro* plantlets. The amplified product hybridized to the cloned BSV probe and the hybridization signal was detected after high stringency washing conditions. The sequence of the PCR product was identical to that of episomal BSV sequence. No product was amplified from nucleic acid extracts of traveller's palm (*Ravenala madagascariensis*) or *Heliconium* sp. nor from samples without

template DNA. Hybridizations using a digoxigenin-labelled, cloned fragment from one BSV isolate indicated an apparent low homology between the probe and the amplification products. Even under moderate wash conditions, the PCR products amplified from some clones that appeared intense on ethidium bromide stained gels produced only faint hybridization signals.

Optimal conditions for amplification were determined. A lower annealing temperature (37 ^{0}C) used in the initial cycles of the PCR program, led to non-specific amplification products being visible on gels. However, only a single PCR product was detected after Southern hybridization. Performing all 40 cycles with an annealing temperature of 55 ^{o}C led to a slight decrease in intensity of the bands observed on agarose gels. An annealing temperature of 60 ^{o}C greatly reduced the intensity of the amplified products from the banana samples tested. Altering the $MgCl_2$ concentration between 4 mM and 2.5 mM had little effect on the observed intensity of amplification products. A concentration of 1 mM $MgCl_2$ resulted in inconsistent amplification of the 664-bp DNA fragment. Under optimal conditions, a product was readily obtained from 0.1 picogram of BSV full length DNA.

Boiling the template DNA with the primers, followed by quenching on ice, increased the sensitivity of detection of ScBV in most sugarcane samples (Braithwaite *et al.*, 1995). This procedure was not necessary for amplification from solutions containing either the cloned BSV virus genome or purified BSV

virions.

Comparison of techniques for BSV-indexing

Twenty plants were examined for typical BSV symptoms and samples taken from leaves showing different appearances. Typical chlorotic or necrotic BSV symptoms were shown by 9 plants (13 symptomatic samples), the remaining 11 being symptomless (23 symptomless samples) (Table 3). ISEM detected BSV in 10 of the 36 samples, while BSV using dot-blot hybridization assay was detected in only 6 of the 36 virus minipreps. However, PCR generated amplification products of the expected size (644 bp) in 32 of 34 virus miniprep samples and produced strong hybridization signals with Southern analysis using a BSV derived probe. The two PCR-negative samples (TMP3x 15108-1 and FHIA-1) were also negative by ISEM (Table 3). These results indicate PCR to be a much more sensitive technique for BSV indexing than the use of symptoms, ISEM or dot-blot hybridization.

IC-PCR and DB-PCR

Both IC-PCR and DB-PCR consistently amplified the BSV-specific 664 bp product from crude leaf extracts or virus minipreps prepared from BSV-infected *Musa* spp. No such product was amplified from crude leaf extract prepared other plant species (Traveller's palm, Tobacco, *Heliconium* sp., Sugar cane) or PCR solutions containing no DNA template.

Pre-treatment of the tubes for 1 hour at 37°C with either 1%

BSA or 5% fat-free skimmed milk powder could reduce the apparent virus binding in both IC- and DB-PCR. In contrast, 1 mg/ml herring sperm DNA as blocking agent had no effect on virus binding.

Musa nuclear intermediate repetitive DNA sequence, the mitochondrial gene *nad*1 and the chloroplast intergenic region DNA could be detected in *Musa* genomic DNA. However they could not be detected in the IC-PCR tubes in which BSV sequences could be detected.

Comparative results of tenfold serial dilutions of crude leaf extract indicated that IC-PCR and DB-PCR are at least as sensitive, and for some samples, more sensitive than standard-PCR probably due to the removal of inhibitory substances by the washing steps in IC- and DB-PCR.

The sensitivity IC-PCR and DB-PCR to detect BSV was compared with that of standard-PCR, ELISA and ISEM using virus minipreps from plants previously indexed by ISEM and ELISA. The sensitivities of the PCR-based methods are comparable to each other and they are all much better than for the other detection methods. IC-PCR distinguishes integrated BSV and episomal BSV sequences and so is useful in the context of the present invention.

DISCUSSION

Despite the concerted efforts of many researchers (Lockhart

and Olszeswki, 1993; Harper *et al.*, 1996; Braithwaite *et al.*, 1995) and international centres (INIBAP, 1995), reliable detection of BSV from infected plant materials has become a serious constraint for the safe movement of improved *Musa* germplasm. Currently, there are no reports on development of protocols for reliable detection of BSV from *in vitro* plantlets that are suitable for germplasm movement. Our results presented in this study demonstrate a PCR technique that detects BSV from such *in vitro* plantlets and is suitable for the large-scale initial screening of *in vitro Musa* germplasm, thus reducing the numbers of potential 'BSV-free' plant materials for further confirmatory indexing.

The approach used here has been to use a "composite" antisera generated against numerous different BSV isolates to trap virus particles and a subsequent PCR with primers based on the sequence of a Nigerian BSV isolate. Alternative degenerate primers are available (Lockhart and Olszeswki, 1993,) for amplification of isolates with widely varying sequence. The washing steps following immuno-capture effectively remove PCR-inhibitory substances improving the reproducibility of results. A detailed comparative analysis of all the reported degenerate primers in combination with restriction analysis, may provide useful information regarding the sequence variability of BSV and the possibility to differentiate and detect all isolates of BSV.

The PCR primers and conditions described here provide a

specific, sensitive method for diagnosing BSV-infected banana/plantain from both *in vitro* propagated or field grown plants. This method is currently being applied for screening and surveying banana germplasm from quarantine plant materials, and in the field to ascertain the full extent and distribution of BSV infection.

Integration

The health status of plant material indexed for BSV solely by PCR may be questioned due to a possibility of BSV DNA integration into *Musa* spp. (LaFleur *et al.*, 1996). Using degenerate primers, integrated sequences of BSV have been detected from *Musa* germplasm collected from all over the world (LaFleur *et al.*, 1996). Our results with PCR of *Musa* DNA using BSV specific primers confirm that essentially all *Musa* cultivars appear to possess integrated BSV sequences. Additional evidence is shown by genomic Southern analysis when carefully isolated very high molecular weight *Musa* DNA hybridises to BSV sequences. The apparently high degree of infection of *Musa* sp. by BSV shown in this study, is mimicked by the widespread infection of sugarcane by the related ScBV (Comstock and Lockhart, 1990, Braithwaite *et al.*, 1995).

Our results show that episomal virus can be specifically detected with high sensitivity and specificity. *Musa* nuclear, mitochondrial or chloroplast DNA which may contain possible BSV integrated sequences are not captured by the anti-sera or by the tubes in which the assay is carried out.

Development of the assay

The ease of sample preparation for this technique is suitable for the handling of large number of samples. Under limited laboratory facility conditions, either antibody-coated or empty Eppendorf tubes may be hand-carried or shipped by collaborating scientists, and the tubes returned back after loading of samples and washing to complete PCR assay. A similar system for detection of viruses by ELISA by mail is reported to be successful (Gaikwad and G. Thottappilly, 1988.)

Dot-blot hybridisation could be a more efficient, economical and technically less demanding detection system for handling large number of samples. However, our results show that ISEM, ELISA and PCR assays were more reliable methods than this assay and confirm the results of Lockhart and Olszeswki, (1993).

ELISA is not sufficiently sensitive or reliable to detect BSV, (a low-titre virus) in infected plants without characteristic symptoms, and its wider application is limited. Nevertheless, ELISA has proved useful to confirm BSV-infection from symptomatic plants. ISEM has proved sensitive enough to detect BSV from plants with low BSV-titre. Our results presented in this study further show that PCR linked to the serological technique provides a rapid, sensitive assay system.

Possible improvements to the current PCR protocols could allow wider application of *Musa* virus-diagnosis. For example, CMV infection of *Musa* has been generally observed under field conditions as sole or mixed infection with BSV. The symptoms caused by CMV are often confused with those caused by BSV. An IC-RT-PCR is being developed for CMV detection with the ultimate aim of developing a simultaneous detection of both BSV and CMV in a single test tube from individual samples.

PCR

Because of its high sensitivity, PCR assay may produce 'false negative or false positive' results. The small-scale DNA extraction procedure allows detection from a very small portion of the tissue. Virus distribution may not be uniform in all tissue portions of a plant, for example in some portions of leaf tissue virus particles may be absent. Therefore, if the assay of a whole plant is based on a single small sample, it is possible that false negative will result. This problem can be reduced, however, by taking multiple samples from different leaves and combining them into a composite samples. Our experiments so far have not shown the virus to be absent from certain parts of a leaf, and leaves are an appropriate tissue for assay.

Experimental errors (e.g. contamination) or non-specific amplification are a possible source of "false" positive results. DNA hybridization of the amplification product with

the homologous probe rules out any possibility of a false positive. These problems can also be minimized by multiple testing of individual plants and the use of independent confirmatory assays (e.g. ISEM) for reliable diagnosis.

Our results have shown a rapid, sensitive and accurate assay for episomal BSV. At present ISEM is the confirmatory diagnostic test especially for international movement of *Musa* germplasm (Diekmann and Putter, 1996). However as the wider application of ISEM for indexing large numbers of samples is limited by labour, equipment requirement and the time required to perform the assay, IC-PCR should be considered as its replacement assay.

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Table 1. *Badnavirus nucleotide homology comparison*

Virus sequence	% nucleotide homology to BSV
CoYMV	42.7
CSSV	49.0
DaBV	47.8
RTBV	40.7
ScBV	51.2

Table 2.

	as-1 sequence	Distance from TATA	TATA sequence
CaMV	cacTGACGtaagggaTGACGcac	34	ctcTATATAAgca
CoYMV	tgaTGATGtcattgaTGACGgcg	176	cctTATTTAAgca
FMV	gtaTTACGaacgcagTGACGaca	22	atcTATTTAAaga
BSV	tagTCACGcacga--TGACCttt	181	ctcTATATAAgga

Table 3 Comparative detection of BSV

Sample No	Symptoms	Result	
		ISEM	PCR
1a	Symptomless	-ve	+ve
1b	Necrotic	+ve	+ve
1c	Symptomless	-ve	+ve
1d	Symptomless	-ve	+ve
2	Symptomless	-ve	-ve
3	Symptomless	-ve	+ve
4	Symptomless	+ve	+ve
5a	Necrotic	-ve	+ve
5b	Symptomless	+ve	+ve
5c	Symptomless	+ve	+ve
6a	Symptomless	+ve	+ve
6b	Necrotic	-ve	+ve
7a	Necrotic	+ve	+ve
7b	Necrotic	+ve	+ve
7c	Chlorotic	+ve	+ve
7d	Chlorotic	+ve	+ve
8	Symptomless	-ve	-ve
9	Symptomless	+ve	
10	Symptomless	-ve	
11a	Symptomless	-ve	+ve
11b	Symptomless	-ve	+ve
11c	Symptomless	-ve	+ve
12a	Chlorotic	-ve	+ve
12b	Necrotic	-ve	+ve
12c	Symptomless	-ve	+ve
13a	Symptomless	-ve	+ve
13b	Symptomless	-ve	+ve
14a	Necrotic	-ve	+ve
14b	Symptomless	-ve	+ve
14c	Symptomless	-ve	+ve
15	Symptomless	-ve	+ve
16	Necrotic	-ve	+ve
17	Necrotic	-ve	+ve
18	Symptomless	-ve	+ve
19	Symptomless	-ve	+ve
20	Chlorotic	+ve	+ve

Table 4

	35S promoter	BSV promoter
Luciferase activity (cpm/μg protein)	1,272,388	7,645,568
	1,372,242	4,004,633
	1,808,389	3,842,321
	833,581	1,458,931
	1,279,123	3,164,783
	872,056	4,139,964
	707,714	2,291,611
	815,308	4,889,694
average:	1,120,100	3,939,688
Glucuronidase activity (pmols 4-MU/min/mg protein)	93,217	29,837
	32,002	13,318
	39,584	16,231
	23,268	8,668
average:	47,018	17,013
Normalized luciferase activity	23.8	232